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Epidermal characteristics of some Western Australian wandoo-woodland species for studies of herbivore diets

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Abstract

Characteristics of the epidermis of plant tissues can provide sufficient differences for separation at least at the generic taxonomic level. The comparisons of known samples with residual fragments of leaf cuticles in faeces provides a non-lethal method of determining the diets of herbivores. This current paper describes the methods employed to establish a voucher series for plants at the Yalanbee Experimental Station for a study of the food resources of the western grey kangaroo.

Three preparation techniques were compared on the wandoo-woodland plant specimens. The chromic acid/nitric acid digestion method gave the best results for most Western Australian native species. For the more mesic native shrub species and the introduced and native grass species, the acetic acid digestion method proved more successful in providing clean specimens of these species within cuticles. Imprint methods generally provided prints of inadequate detail for use in identifying the residual fragments from the faecal remains.

A diagnostic key using epidermal characteristics provided a method to extend the techniques of faecal fragment identification to use by non-specialised technical assistants.

Introduction

Studies of the diets of herbivores date from at least the mid-eighteenth century when, according to Tribe (1950), Linnaeus reported on the preference shown by farm animals for certain plant species. For almost the next two centuries the techniques used for the study of herbivore diets were dominated by those used for domestic animals in enclosed situations. Most of these techniques had practical limitations or were impossible to apply to open grazing and other less intensive farming practices. A developing interest in the food preferences of game and other non-domesticated animals led to new techniques for the study of food habits. One of these techniques was the identification of fragments of leaf epidermis from faeces (Baumgartner and Martin 1939, Dusi 1949). Except for vascular bundles, fibrous strengthening material and cutinized epidermal tissue, little plant material remains recognizable in faecal material (Phillipson 1952). Only the cuticle and its epidermal imprint has sufficient characteristics to be identified as belonging to a specific species.

The literature on dietary studies utilizing epidermal characters has been accumulating over the past 40 years (Dusi 1949, Stewart 1965, Kok and Van der Schijff 1973) and detailed accounts of the characteristics of the epidermal structures of monocotyledons (Metcalfe 1960, Clifford and Watson 1977) and dicotyledons (Metcalfe and Chalk 1950) have provided extensive background information. However, it was not until Storr (1961), Kiley (1966) and Stewart (1965, 1967) that the full potential of faecal analysis was realised in determining the feeding habits of wild and rare animals. Studies of the dietary preference of Australian macropods are limited (Kirkpatrick 1965, Griffiths and Barker 1966, Griffiths *et al.* 1974, Davis and Ellis 1979) and such studies in Western Australia are restricted to those of Storr (1961, 1964) on the quokka. Faecal analysis was used to gain further knowledge about the diet of the western grey kangaroo at the CSIRO Yalanbee Experimental Station in the wandoo (*Eucalyptus wandoo* Blakely) woodland-pasture landscapes near Bakers Hill. This method was the only practical option available, because the animals of the experimental station were part of a population dynamics study and could not therefore be killed to determine dietary resources through analysis of stomach contents. Because no previous work on the diet of the grey kangaroo had been attempted in the wandoo woodland, epidermal tissue vouchers had to be initially developed. The following report describes the development of methods for the preparation of plant epidermi, the nomenclature of epidermal characteristics for the series of voucher specimens, and the diagnostic key used in the western grey kangaroo dietary study (Halford *et al.* 1984.)

Epidermial tissue preparation

A number of techniques have been developed to prepare epidermal tissue for microscopic analysis. Once high-quality voucher specimens are available, the subsequent identification of epidermal fragments in animal faeces is made possible. These preparation techniques can basically be categorised into direct and indirect methods. Direct methods usually involve the removal of the leaf surface from the remainder of the plant material by bacterial disintegration, mechanical scraping, peeling or chemical maceration. Bacterial disintegration of the tissue (Louw *et al.* 1949, Skoss 1955) involves the breakdown of the tissue below the cuticle using certain microorganisms which digest the material with an enzyme complex of cellulases, hemicellulases and pectinases. Following a period of time the cuticle is mechanically lifted from the partially digested tissue. The major difficulty of the method is the time (often 14 days) required to obtain material and the specialized media requirements and anaerobic growing conditions for the bacteria.

Epidermal peeling (Dusi 1949) and mechanical scraping (Metcalfe 1960, Clarke 1960) involve the removal of the epidermal layer directly, as one might remove the peel of a ripe plum, or by gradually scraping the leaf tissue from the cuticle. These methods are time-consuming and difficult to apply where the epidermis is thin and closely associated with fibrous material, or where the leaves are ridged and furrowed, as in many Australian sclerophylls. The chemical maceration techniques utilize a variety of solutions to macerate tissue in order to free the cuticular layer membrane. Discussion and literature references for many maccrating techniques are available (e.g. Sinclair and Sharma 1971). Generally these methods use mixtures and various concentrations of several weak acids, or hydrogen peroxide and a base as potassium hydroxide. Most of these methods are adequate in clearing the cuticle fragments from any remaining mesophyll or fibrous material, but the concentration of solutions, timing of various digestion periods and subsequent stain techniques often vary with the type of leaf material to be prepared. The major advantage of the direct method is that they can give a clear representation of the cuticle; however, the procedures may be time-consuming.

The indirect methods involve applications of liquid plastics or latexes which, upon drying to a thin, transparent film, can be peeled off and examined under a microscope. The materials used include Duco cement (William 1973), acrylic plastics (Horanic and Gardner 1967), cellulose acetates, Bennett and Furmidge 1956), polyvinyl alcohol (Mueller *et al.* 1954), mucilage and latex (Shah and Gopal 1969), and silicone rubber (Idle 1969). Indirect methods are generally much quicker and simpler to apply than the direct methods, but the quality of the epidermal reproduction is usually inferior.

Materials and methods

Three methods were examined to ascertain the most successful method for species of the wandoo-woodland vegetation. These included the indirect method of Horanic and Gardner (1967) and the direct methods of Storr (1961) and Jain (1976). In the Horanic and Gardner method, a 6:6 mixture of Rhoplex AC-33 acrylic polymer emulsion was initially applied to the surface of the leaf and allowed to dry for 10 minutes. Two additional coats using full-strength Rhoplex were applied at 10 minute intervals and allowed to dry for 40 minutes. The emulsion was then removed and transferred to a microscope slide. Generally the imprints did not give adequate representation of the epidermi of a large number of the sclerophylls. It was unable to show any detail of ridged or deeply furrowed leaf surfaces and abaxial surfaces of leaves with revolute margins. The preparation was difficult to apply to terete leaves because the resulting film was curved and difficult to view under the light microscope or photograph.

Applying the method or Storr (1961), leaves were cut into 5 mm squares or, if leaves were small, they were trimmed along one edge to facilitate epidermal removal. The material was placed in a solution of equal parts 10% chromic acid and 10% nitric acid and carefully boiled in a fume hood until epidermal separation occurred. A dilute KOH solution was used to rinse the tissue before the cuticles were removed from remaining fibre and vascular tissue. Pieces of the epidermis were transferred through a graded series of ethanol from 10-95%, then stained with 0.1% gentian violet in 95% ethanol. Staining time varied with the material and ranged from 30 sec. to to 24 h. The stained tissue was transferred to absolute ethanol and mounted in euparal. Storr's technique gave good results for a broad range of the Western Australian species, excepting those species which had thin cuticles, where features were difficult to distinguish.

Using the method of Jain (1976), material was cut into 5 mm squares and allowed to stand in a 30% glacial acetic acid in water solution at 80°C for 24 h. The material was then washed with water, cleared of remaining fibrous tissue and dehydrated through the series of ethanol solutions to 95%, and stained in 0.1% gentian violet in 95% ethanol before mounting in euparal. Jain's method did not thoroughly clear the epidermis of the more sclerotic leaves, such as *Hakea trifurcata* and *Acacia celastrifolia*. It did, however, prove to be very successful in producing clean epidermi from grasses and the native species with more mesic leaves such as *Phyllanthus calycinus*.

Microphotographs of the best preparations were made at low magnification (30x). The photographs were taken with a Zeiss photo-microscope using Microfile Pan film.

To analyze the faecal material, a dried pellet was gently boiled for 15 min in a solution of equal parts 10% chromic acid and 10% nitric acid. After maceration, the material was filtered and rinsed several times with a dilute KOH solution, then stained with a 0.5% solution of gentian violet in 95% ethanol for 48 h. The stained fragments were mounted onto microscope slides in euparal. The fragment material could then be compared to the vouchers of suspected diet plants to determine the food resources actually ingested.

Epidermial character nomenclature

The epidermis of leaves has a number of characteristics which are useful in identification and classification of plants. In the field of palaeobotany, microscopic features of the epidermis (as they are reflected in the cuticle), have long been recognized as an aid in the study of fossil leaves (Bornemann 1856 cited by Dilcher 1974). Stace (1965) has shown how leaf epidermal features can be used to support other taxonomic aids, especially in the classification of the Combretaceae. More recently, Bongers (1973) used epidermal character in the taxonomy of the Winteraceae. Also epidermal characters have been applied as a safeguard against fraudulence in commercial stock feed (Parkinson and Fielding 1930 cited by Martin 1955), and police departments have found the epidermis useful in plant identification (Dilcher 1974). Useful epidermal characters used in the identification of fragmentary material include: cell orientation, stoma complexes, dermal appendex, and other epidermal cells.

Cell orientation

Storr (1961) in his faecal analysis work found the orientation of the epidermal cells to be a very useful diagnostic feature. The distinctive orientation of the epidermal cells along the longitudinal axis of the leaf is commonly observed in monocotyledons (Fig 1a), and is rare among dicotyledons where these cells are more irregularly distributed (Fig 1b). This basic pattern difference readily discriminates between these two large groups and can be beneficial in distinguishing relative proportions of grazing and browsing material eaten by herbivores.

Stoma complexes

The appearance of the stomata, especially with reference to the nature and orientation of neighbouring cells, is a frequently used taxonomic character (Metcalfe and Chalk 1950, Van Cotthem 1970). Dilcher (1974) has provided a series of terms relevant to the stomatal cell complexes which have proven helpful in the identification of epidermal tissue fragments (Fig. 1). The stomata complex of monocotyledons are commonly of the paracytic type. However, types such as tetracytic and anocytic have also been observed (Metcalfe 1961, Stebbins and Khush 1961). An added feature of the stomata complexes in monocotyledons is the shape of the subsidiary cells. These cells range from triangular to parallel-sided forms through intermediates to dome-shaped cells (Metcalfe and Chalk 1950). A species usually has a mixture of domes with either triangular or parallel-sided types, but it is very unusual to find triangular and parallel-sided types together (Clifford and Watson 1977).

In dicotyledons, the more common stomata complex types are anomocytic, anisocytic, paracytic and diacytic. Storr (1961) suggested that the first three are the most commonly observed types in Australian species. Potential variation does exist and Metcalfe (1960) warns that stomatal types of more than one kind sometimes occur together on the same leaf surface, or stomatal complexes on the upper and lower surfaces may not be alike.

Storr (1961) also suggests that stomata pore orientation may be useful as a diagnostic feature, but this appears to be of restricted use.

Dermal Appendages

All outgrowths of the epidermis are categorized under the term 'dermal appendage'. There is an enormous diversity of external appendages in the dicotyledons, but this is not exhibited as extensively in the monocotyledons (Metcalfe 1960). Radford *et al.* (1974) summarizes the types of epidermal appendages under three main headings including 'simple' trichomes which can be uniseriate or multiseriate, compound trichomes of several forms and glandular trichomes. The features of length, size and density of the trichomes are more liable to vary with environment than the occurrence of different kinds (Sharma 1975). These characters should be avoided because of their questionable value for identifying epidermal fragments of different species. The presence or absence, however, can be of assistance. Gray *et al.* (1969) have demonstrated that the presence or absence of hairs between two closely related varieties of *Danthonia* was not environmentally induced, as the hairs were found to persist under a variety of controlled environments and transplant studies.

Other epidermal cells

The epidermal areas between the leaf veins (intercostal) and the tissue over the veins (costal) in monocotyledons sometimes have two cell types distinguishable by their size. Much elongated cells called 'long cells' tend to occur in intercostal regions, while more equidimensional cells called 'short cells' tend to occur only in the costal regions. In some grasses such as *Bromus* the short cells are absent (Metcalfe 1960). In others, such as *Coelachne* and *Mibora,* the distinctions tend to break down, which reduces their value as diagnostic features. Short cells also may contain a silica body or walls which give a cork reaction and the number occurring together or the arrangement may be of diagnostic value. The walls of the long cells of grasses can vary from straight and clear to very sinuous and clearly pitted. Also, the relative length and breadth can be of some value, but is subject to considerable variation.

The cells of dicotyledons are less regular than those found in the monocotyledons. The abaxial surface in some dicotyledons may be completely devoid of trichomes or stomata. There is generally, however, sufficient difference in the cells of different species to be of potential taxonomic value when looking at a limited number of species, as in faecal analysis study at the Yalanbee Experimental Station (Halford *et al.* 1984).

Intraspecific variation

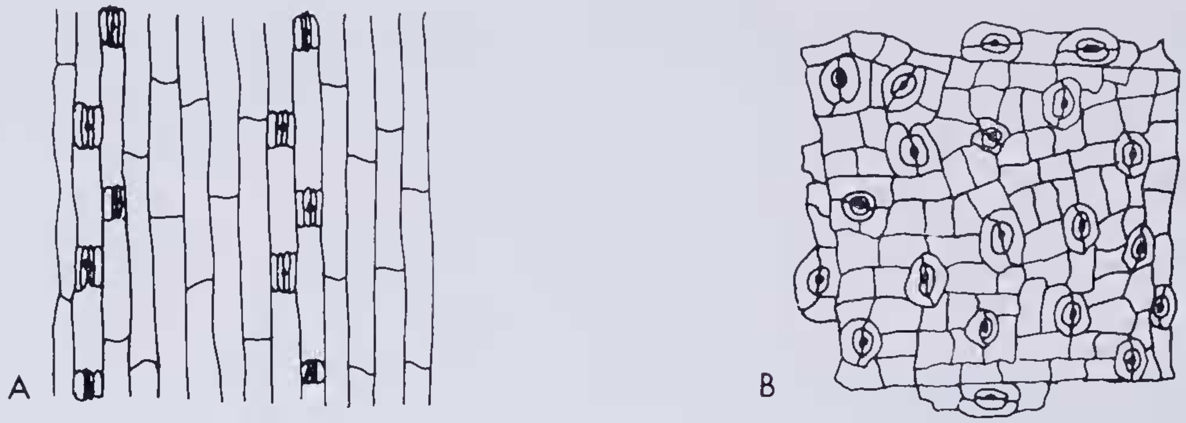
It is important to differentiate environmentally modifiable features from those controlled genetically. Esau (1960) has noted that features such as the number of stomata per unit area, and whether guard cells are raised or lowered when compared to other epidermal cells, are so variable that they are of little taxonomic value. Characteristics such as a stomata frequency, epidermal cell frequencies and trichome densities generally vary with growing conditions. On the other hand, characteristics such as the type of stomatal complexes, distribution pattern of trichomes and types of trichomes tend not to vary with the environmental conditions and are, therefore, of greater value as diagnostic features in studies examining dietary preferences.

Wandoo-woodland vouchers

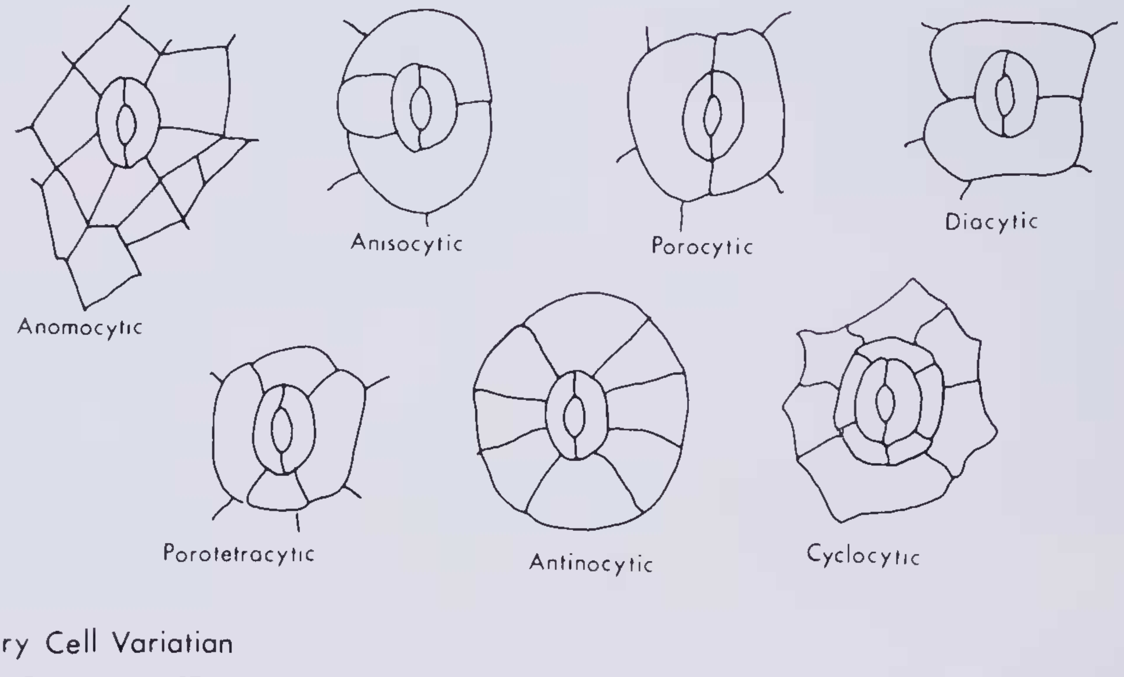
Of these species of the natural woodland regions and the pastures of the Yalanbee Experimental Station, slides of 60 species were produced of suitable quality for comparisons to the fragmentary material from the faeces of the grey kangaroos resident in the area. The voucher slides, enlarged photographs and descriptions of the important epidermal features of these species were used to prepare the diagnostic key to the more common species found in the faecal samples (Table 1).

All monocotyledons from the study area had stomatal apertures along the longitudinal axis of the leaf. Examples of Australian natives were *Neurachne alopecuroides, Lepidosperma tenue* and *Loxocarya flexuosa.* The introduced Mediterranean grasses of the pasture area also exhibited this pattern. Although this feature was rarer in the dicotyledons, it was observed in *Calothamnus sanguineus, Adenanthos cygnorum, Astroloma pallidum* and *Stackhousia hueglii.* In *Casuarina* the stomatal aperture was perpendicular to the long axis of the needle. No other dicotyledon showed regular orientation of the stomatal apertures.

Epidermal Cell Orientation



Stomatal Complex Types



Subsidiary Cell Variation

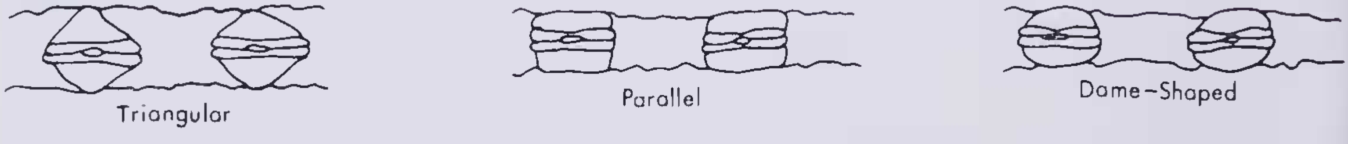


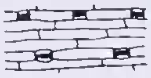
Figure 1.—Epidermal tissue variation. Epidermal cell orientation in: (A) a monocotyledon (*Bromus rigidus*) shownig longitudinal orientation of epidermal cells and (B) a dicotyledon (*Acacia celastrifolia*) showing irregular orientation. Stomatal complex type nomenclature of angiosperm leaves (after Radford *et al.* 1974) and subsidiary cell variation of monocotyledons (after Metcalfe 1960).

The nature and orientation of the subsidiary cells of the stomata proved to be especially diagnostic in the separation of the dicotyledonous species at the Yalanbee Experimental Station. The anomocytic stomatal arrangement, where cells surrounding the stomata are indistinguishable from normal epidermal cells, was found in *Hibbertia hypericoides, H. montana, Astroloma pallidum* and *Olearia rudis.* The anisocytic arrangement of three distinct subsidiary cells enclosing the stomata was observed in *Boronia crenulata, Hemigenia canescens* and *H. pimelifolia.* The paracytic arrangement of two

Table 1.

Key to the identification of selected native species in the Yalanbee Experiment Station woodland region using epidermal characteristics.

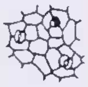
A. Epidermal cells longitudinally orientated



B. Epidermal cells rectangular (length:width ratio >> 1 : 1) .... Conostylis setigera

BB. Epidermal cells more-or-less isodiametric (length:width ratio = 1:1) .... Loxocarya flexuosa

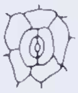
AA. Epidermal cells usually irregular in orientation



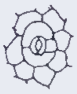
B. Trichomes or hair present

C. Trichomes scattered

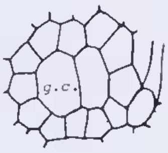
D. Stomatal cell complexes paracytic



...... or anisocytic ......



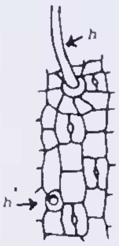
E. Epidermis with glandular cells (g.c.) surrounded by ring of typical epidermal cells ....



..... Leptospermum erubescens

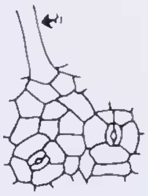
EE. Epidermis lacking glandular cells

F. Stomatal complexes in ± linear arrangement, hairs (h) easily broken (h')during preparation........



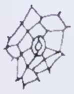
. Adenanthos cygnorum

FF. Stomatal complexes irregular in arrangement, hairs robust and persistent ....

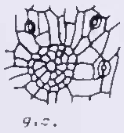


.... Boronia crenulata

DD. Obviously subsidiary cells absent (i.e. anomocytic)
(NB. Calothamnus sanguineus may show paracytic forms also)

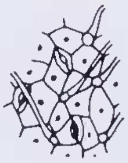


E. Glandular cells present.....



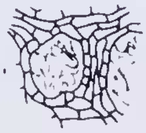
.... Calothamnus sanguineus

EE. Glandular cells absent, epidermal cells with papillose projection common ....



.... Oxylobium parvifolium

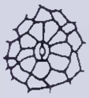
CC. Trichomes clustered in circular depressions within the intercostal regions ....



.... Dryanora spp.

BB. TRICHOMES ABSENT

C. STOMATAL CELL COMPLEXES ANTINOCYTIC ....



.... HAKEA SPP.

CC. STOMATAL CELL COMPLEXS NOT ACTINOCYTIC

D. STOMATAL COMPLEXES ARRANGED IN DEFINITE BANDS

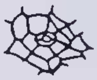
E. STOMATAL BANDS • 2X WIDTH OF COSTAL REGIONS,
STOMATA IN ONLY A FEW LINES .... ASTROLOMA PALLIDUM

EE. STOMATAL BANDS ≫ 2X WIDTH OF COSTAL REGION,
IN NUMEROUS LINES ....CONOSPERMUM GLUMACEUM

DD. STOMATA COMPLEXES NOT IN BANDS

E. STOMATAL CELL COMPLEXES CELLS ANOMOCYTIC OR ANISOCYTIC (SEE BELOW)

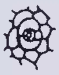
F. SUBSIDIARY CELLS ANOMOCYTIC



G. GUARD CELLS SUNKEN .... HOVEA CHORIZEMIFOLIA

GG. GUARD CELLS NOT SUNKEN .... GOMPHOLOBIUM MARGINATUM

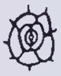
FF. GUARD CELL COMPLEXES ANISOCYTIC



G. GUARD CELLS SUNKEN .... DAVIESIA CORDATA

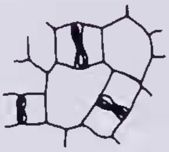
GG. GUARD CELLS NOT SUNKEN .... ANTHOCERCIS LITTOREA

EE. STOMATAL COMPLEXES PARACYTIC



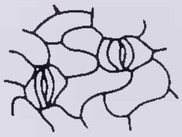
F. SUBSIDIARY CELLS ± EQUAL IN SIZE

G. EPIDERMAL CELL WALLS ± STRAIGHT, GUARD CELLS
± SQUARES IN OUTLINE ....



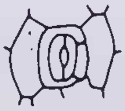
... GASTROLOBIUM SPP.

GG. EPIDERMAL CELL WALLS ROUNDED OR SLIGHTLY
UNDULATING, SUBSIDIARY CELLS ± TRIANGLAR...



. SYNAPHAEA PETIOLARIS

FF. SUBSIDIARY CELLS UNEQUAL IN SIZE ....



.... ACACIA CELASTRIFOLIA

subsidiary cells parallel to the long axis of the stomata was recorded in all species of the genus *Gastrolobium* and all of the monocotyledon species studied. *Adenanthos cygnorum*, and all species of *Casuarina* also had the paracytic arrangement. All species of the genus *Hakea* examined had the antinocytic arrangement of stomata surrounded by radially elongated subsidiary cells. *Calothamnus sanguineus* was found to have a combination of anomocytic and paracytic types. In other species, such as *Grevillea pilulifera* and all the *Dryandra* species prepared, the stomata were not discernible, due to a dense mat of trichomes covering the stomata.

Stomatal distribution in the plants of the Yalanbee Experimental Station study site also provided a diagnostic character. A regular distribution of the stomata over the epidermal surface was observed in *Acacia celastrifolia*, *Calothamnus sanguineus*, *Adenanthos cygnorum* and *Hakea undulata*. Certain species had stomata arranged in distinct repeated patterns. This included the stomata of *Lepidosperma tenue*, *L. scabrum* and *L. angustatum* where the stomata were arranged in rows oriented along the longitudinal axis. In most of the species of the grasses, the stomata occurred in well-defined longitudinal bands in the intercostal zones. Each intercostal zone included one or more stomatal band depending on the species.

Stomata in some species tended to be associated with epidermal pits and furrows. The stomata of the *Dryandra* species studied were confined to depressions on the abaxial surface of leaves. The stomata of leaves of *Lomandra caespitosa* and *L. effusa* and the stems of *Opercularia vaginata*, *Casuarina humilis*, *C. huegeliana* and *Dampiera lavandulacea* were limited to longitudinal grooves.

Trichomes occurred in many of the species sampled from the wandoo woodland. Simple unicellular trichomes were present in *Boronia crenulata*, *Melaleuca holosericea*, *Leptospermum erubescens*, and *Hemigenia canescens*. *Adenanthos cygnorum* had simple unicellular trichomes but was distinguished by a large, flat basal cell to the trichome. *Hibbertia hypericoides* had sessile stellate hairs and *Hemigenia canescens* had glandular and bicellular hairs. All of the species of *Dryandra* in the study had lociform trichomes densely covering the abaxial surface. *Calothamnus sanguineus* had setaceous trichomes, but these tended to break down during preparation of the epidermi. Several species had papillose projections from the epidermal cells. These included *Gastrolobium calycinum* and all of the species of *Casuarina examined*. The grasses tended to have mostly unicellular trichomes, which were not valuable in the identification of individual species.

Shape of the epidermal cells was helpful in distinguishing monocotyledons from dicotyledons. The cells of the monocotyledons were typically rectangular, with the length parallel to the longitudinal axis of the leaf. Some intermixed of the monocotyledons, however, were square. The dicotyledons varied from the isodiametrical cells as in *Dryandra sessilis* to more rectangular cells as in *Opercularia vaginata*. Irregular shaped cells in *Calothamnus* and occasional paired cells separated by a sinuous wall in *Calothamnus* and *Leptospermum* were diagnostic. *Loxocarya flexuosa*, *Astroloma pallidum* and some of the grasses were also distinguishable from other species by sinuous epidermal cell walls. Other plants had typically straight walls with varying degrees of curvature at the corners.

In general, most of the plant epidermi from the Yalanbee Experimental Station study area had enough difference to allow identification to genera. However, within some genera, such as *Hakea*, the identification of epidermal fragments to a single species was not always possible. Even so, through careful preparation of faecal material and voucher specimens, it has been possible to develop a basis for studying the dietary preferences of the western grey kangaroo in the wandoo woodland-pasture landscapes of the Yalanbee Experimental Station. With the aid of the key it is possible to examine material rapidly and identify the common species being grazed.

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